(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau

(43) International Publication Date
6 November 2008 (06.11.2008)

PCT

(10) International Publication Number
WO 2008/133801 A1

(51) International Patent Classification:
A61K 31/70 (2006.01) *C08B 37/00* (2006.01)

(21) International Application Number:
PCT/US2008/004546

(22) International Filing Date: 9 April 2008 (09.04.2008)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
11/785,988 23 April 2007 (23.04.2007) US

(71) Applicant *(for all designated States except US)*: **ALBERT EINSTEIN COLLEGE OF MEDICINE OF YESHIVA UNIVERSITY** [US/US]; 1300 Morris Park, Bronx, NY 10461 (US).

(72) Inventor; and
(75) Inventor/Applicant *(for US only)*: **PORCELLI, Steven, A.** [US/US]; 86 Reynold Street, Bronx, NY 10464 (US).

(74) Agents: **DIETZE, Paul, E.** et al.; Kenyon & Kenyon LLP, 1500 K Street, N.W., Suite 700, Washington, DC 20005 (US).

(81) Designated States *(unless otherwise indicated, for every kind of national protection available)*: AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, SV, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM, ZW.

(84) Designated States *(unless otherwise indicated, for every kind of regional protection available)*: ARIPO (BW, GH, GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MT, NL, NO, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Declarations under Rule 4.17:
— *as to applicant's entitlement to apply for and be granted a patent (Rule 4.17(ii))*
— *as to the applicant's entitlement to claim the priority of the earlier application (Rule 4.17(iii))*

Published:
— *with international search report*

WO 2008/133801 A1

(54) Title: CERAMIDE DERIVATIVES AS MODULATORS OF IMMUNITY AND AUTOIMMUNITY

(57) Abstract: α-Galactosylceramides and glycosylceramides ("ceramide-like glycolipids") that modulate NK T cells. The ceramide-like glycolipids vary in the cytokines induced in NK T cells and vary in the antigen-presenting cells that are capable of efficiently presenting the compounds to NK T cells. Pharmaceutical compositions of the ceramide-like glycolipids are provided, as are pharmaceutical compositions of the ceramide-like glycolipids combined with dendritic cells. Methods utilizing the ceramide-like glycolipids in vaccines, to activate NK T cells, to stimulate the immune system, and to treat mammals are also provided. The invention also provides methods of evaluating a compound for its ability to activate an NK T cell in the presence of a cell expressing a CDId protein.

presence of more than one cell type that expresses a CD1d protein and evaluating whether the NK T cell is activated.

The invention is also directed to methods of treating or preventing an autoimmune disease, cancer, or an infection in a mammal. The methods comprise administering to the mammal the above-described pharmaceutical composition.

BRIEF DESCRIPTION OF THE DRAWINGS

FIG. 1 shows the structure of the αGalCer KRN7000, a specific activator of CD1d-restricted NK T cells. KRN7000 is ((2S, 3S, 4R)-1-O-(α-D-galactopyranosyl)-N-hexacosanoyl-2-amino-1,3,4-octadecanetriol). It contains a C18 phytosphingosine base and a C26 fatty acyl group. See U.S. Patent No. 5,780,441.

FIG. 2 shows data from Hong et al. (2001) that KRN7000 prevents development of type 1 diabetes in NOD mice. Mice received twice weekly injection with KRN7000 beginning at 4 weeks of age. Top graph shows reduction in incidence of diabetes from 75% to approximately 5%. Bottom graph shows that this effect is not seen in NOD mice that carry a knockout locus for CD1. These mice do not have CD1-restricted NK T cells, which are required for all of the known effects of KRN7000.

FIG. 3 shows the structure of OCH, an analogue of αGalCer with enhanced ability to suppress autoimmune inflammation in mouse EAE. This differs from the KRN7000 structure in that it has a shortened C9 sphingosine base (as opposed to a C18 sphingosine base).

FIG. 4 shows the core structure and coupling reaction for synthesis of amino-substituted ceramide-like glycolipids identified in the present invention.

FIG. 5. Panel A shows experimental results of bioactivity screens of ceramide-like glycolipids produced by combinatorial synthesis. NK T hybridoma DN32.D3 was cultured with CD1d-transfected RMA-S cells in microtiter plate wells. Each ceramide-like glycolipid was titrated over a concentration range from 0.5 - 500 nM, and supernatants were harvested after 24 hours for measurement of IL-2 release. Units of activity were calculated as the reciprocal of the concentration of ceramide-like glycolipid required to give a half maximal release of IL-2, and all values were normalized to the activity of KRN7000 (defined as 1 Unit). The dotted line indicates the level of activity for KRN7000. The structures of two ceramide-like glycolipids with markedly elevated activity relative to KRN7000 are shown. Panel B shows the structures of the ceramide-like glycolipids tested in the experiments described in Panel A and Table 1. Panel C shows a graphical representation of the results from Table 1. Panel D shows stimulation of CD1d-dependent

proliferation by DB02-1, an α-glucosyl ceramide identical to DB01-1, except with a glucose replacing the galactose of DB01-1.

FIG. 6 shows experimental results of the differential presentation of ceramide-like glycolipids by various presenting cell types. IL-2 production by NK T hybridoma DN32.D3 in response to eight selected ceramide-like glycolipids is shown, using three different cell types as antigen presenting cells (APCs) (top). Structures of the amino linked side chains in each of the ceramide-like glycolipids used in this experiment are shown on the bottom.

FIG. 7 shows the effects of fatty acid chain length on the potency of ceramide-like glycolipids. Ceramide-like glycolipids with the indicated chain lengths were tested for stimulation of NK T hybridoma DN32.D3 using RMA-S/CD1d as antigen presenting cells (APCs).

FIG. 8 shows the effects of fatty acid chain unsaturations on potency of C20 FA ceramide-like glycolipids. Ceramide-like glycolipids with the indicated chain lengths and indicated numbers of double bonds were tested for stimulation of NK T hybridoma DN32.D3 as in FIG. 7.

FIG. 9 shows the selective stimulation of IL-4 production *in vivo* in mice by ceramide-like glycolipids DB03-4 and DB03-5. Serum levels of IL-4 and IFNγ after a single injection of DB01-1, DB03-4, or DB03-5 are shown. C57BL/6 mice (11-13 weeks old) were given a single i.p. injection of 4.8 nanomoles of the compounds or phosphate buffered saline (PBS)/vehicle control. Serum cytokine levels were measured 2 and 20 hours later by capture ELISA. Bars show means of three mice, with standard deviation. Note that DBO1-1 has nearly identical structure to KRN7000 (C24 fatty acid as compared to C26) and has activity that is indistinguishable from KRN7000 in multiple bioassays. We use DB01-1 as a "KRN7000 mimic" because it has been synthesized by our group and is readily available for our studies, and KRN7000 was unavailable due to license restrictions.

FIG. 10 shows experimental results establishing that DB03-4 and DB03-5 are superior to KRN7000 for prevention of diabetes in NOD mice. Cohorts of 6-8 female NOD mice were treated with placebo (vehicle) or DB01-1 (indicated as KRN7000), DB03-4 or DB03-5 injections once weekly beginning at 4-5 weeks of age. Treatment was discontinued after 5 injections (DB03-4) or 7 injections (DB03-5). Top graphs show incidence of glycosuria, and bottom graphs show survival in each cohort.

FIG. 11 shows graphs of experimental results showing that various ceramide-like glycolipids of the present invention can stimulate expression of CD40L (CD154).

FIG 12 is a graphical representation showing IFNγ induction by DB04-1 (KRN-7000), DB05-9, DB05-10, DB05-11, DB05-12, DB05-14, DB05-15, DB05-16, and DB05-17, determined as described in the experimental section.

FIG 13 is a graphical representation showing IL-4 induction by DB04-1 (KRN-7000), DB05-9, DB05-10, DB05-11, DB05-12, DB05-14, DB05-15, DB05-16, and DB05-17, determined as described in the experimental section.

FIG 14 is a graphical representation showing the stimulation of CD1d-dependent proliferation by DB04-1 (KRN-7000), DB05-9, DB05-10, DB05-11, DB05-12, DB05-14, DB05-15, DB05-16, and DB05-17 as described in the experimental section.

FIG 15 is a graphical representation of maximum IFNγ production and maximum IL-4 production when cells are treated with 500 nm of DB04-1 (KRN-7000), DB05-9, DB05-10, DB05-11, DB05-12, DB05-14, DB05-15, DB05-16, and DB05-17 as described in the experimental section.

FIG 16 is a graphical representation of the ratio of IL-4 production to IFNγ production for compounds DB04-1 (KRN-7000), DB05-9, DB05-10, DB05-11, DB05-12, DB05-14, DB05-15, DB05-16, and DB05-17 as described in the experimental section.

DETAILED DESCRIPTION OF THE INVENTION

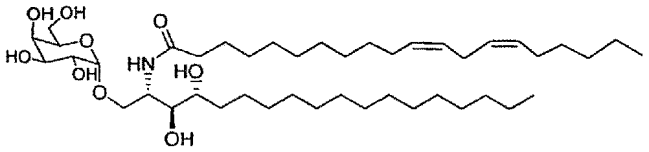
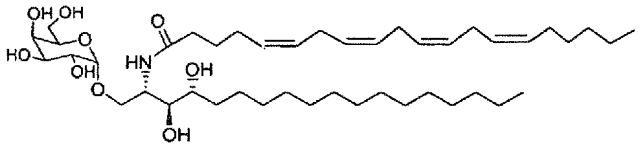
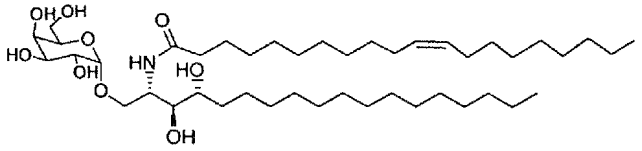
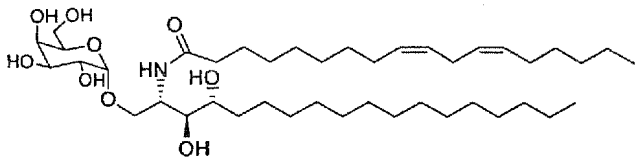
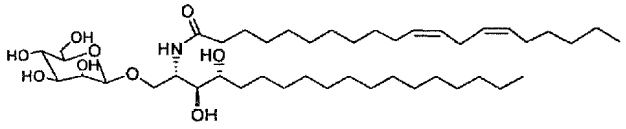
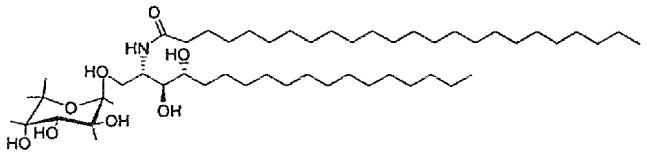
The present invention is based on the discovery that various ceramide-like glycolipids, *i.e.*, α-galactosylceramides or other α-glycosylceramides, ("the ceramide-like glycolipids") are capable of modulating NK T cells, particularly variants in the moiety that is a fatty acid in KRN7000. The invention is also based on the discovery that the ceramide-like glycolipids differ in the type of cell that efficiently presents them, and that they can induce varied cytokine profile when used to activate NK T cells.

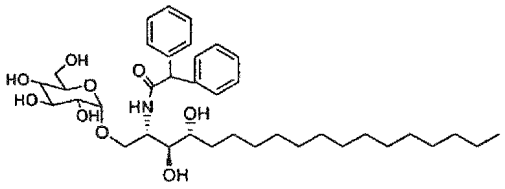
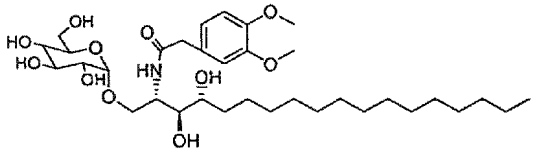
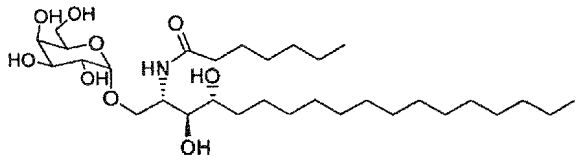
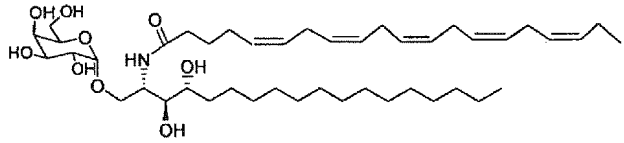
In one embodiment, the ceramide-like glycolipids activate cytokine production by the NK T cells. In another embodiment, the ceramide-like glycolipids suppress cytokine production by the NK T cells. In yet another embodiment, the ceramide-like glycolipids change the ratio of cytokines produced by the NK T cells.

Definitions:

The phrase "presents the compound," as used herein, means a cell binds the compound on the surface of the cell to provide a complex that causes the modulation of NK T cells.

The phrase "efficiently presents the compound," as used herein, means that cells will bind the compound on their surface to provide a complex that causes the modulation of NK T cells when the compound is present at a concentration of less than about 1 μM.

Compound	Molecular Weight	Structure
DB03-4	770.13	
DB03-5	766.13	
DB03-3	772.15	
DB03-10	742.08	
DB04-9	770.13	
DB03-8	886.42	

YTC03-17	705.96	
YTC03-24	657.83	
YTC03-25	591.82	
DB03-6	764.13	

In vivo activity refers to activity in mice (see Example).
In vitro Activity refers to activity in a murine cell assay system (see Example).

Each of the DB03-3, DB03-4, DB03-5, DB03-7, DB03-8, DB03-9, DB03-10, and YTC03-17 show in vitro activity at modulating cytokines using a murine cell assay system (see Example) and, for several of the compounds, activity has also been shown in a human in vitro NKT cell assay system. For example, DB03-4 and DB03-5 are active in stimulation of human NKT cell clones in vitro, and elicits proliferative responses and cytokine secretion when evaluated using culture systems previously established in the literature (*See, e.g.*, Spada FM, Sugita M, Watts GFM, Koezuka Y, and Porcelli SA. Low expression but potent antigen presenting function of CD1d on monocyte lineage cells, *Eur. J. Immunol.*, 30:3468-3477 (2000), and Spada FM, Koezuka Y, Porcelli SA, CD1d-restricted recognition of synthetic glycolipid antigens by human NK T cells. *J Exp Med*; 188:1529-1534 (1998), see also, Lee PT et al., *J Clin Invest.*, 2002 Sep;110(6):793-800. In addition, these compounds can be used to create glycolipid human CD1d tetramers that bind strongly to human NKT cells in normal blood specimens, indicating that these glycolipids, when presented by human CD1d, are avidly recognized by the T cell antigen receptors of human NKT cells (methods for CD1d tetramer production and application to studying NKT cells

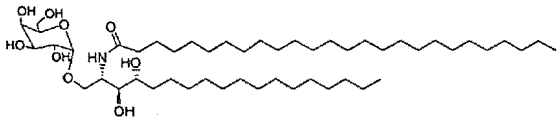
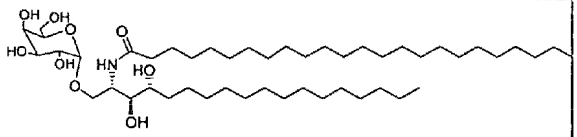
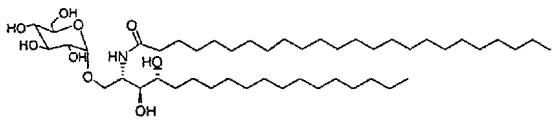
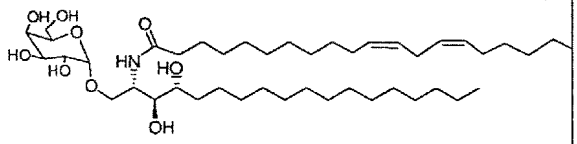
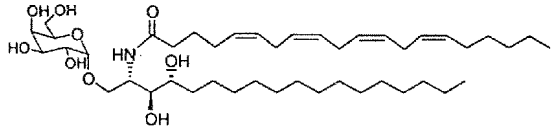
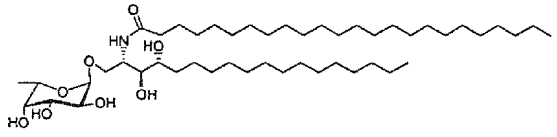
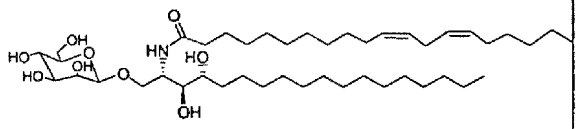
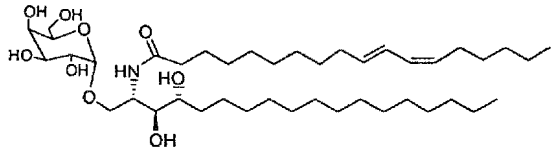
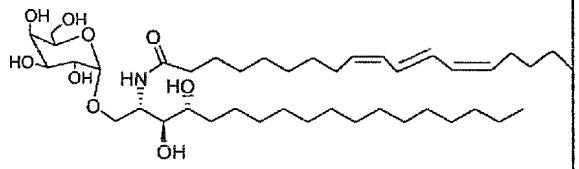
are described in Yu KOA, Im JS, Molano A, Dutronc Y, Illarionov PA, Forestier C, Fujiwara N, Arias I, Miyake S, Yamamura T, Chang Y-T, Besra GS, Porcelli SA, Modulation of CD1d-restricted NKT cell responses using N-acyl variants of α-galactosylceramides, Proc. Nat. Acad. Sci. (USA), 102:3383-8 (2005)).

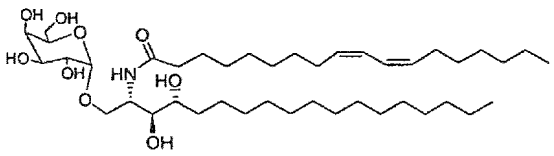
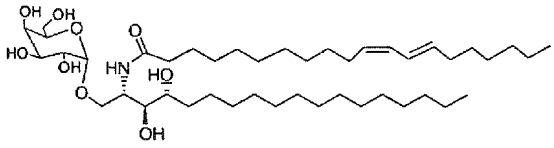
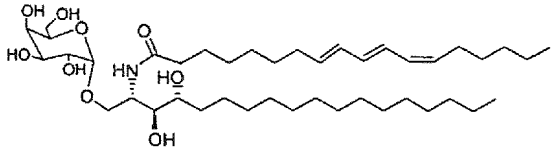
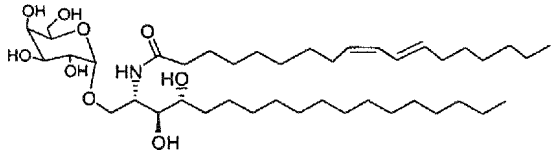
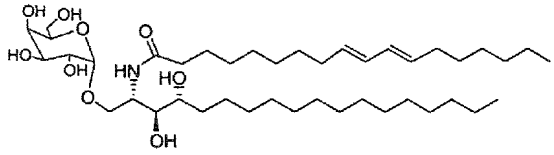
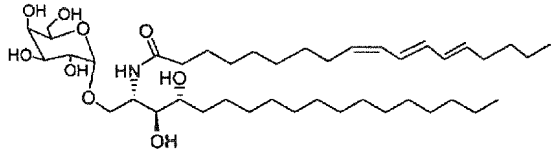
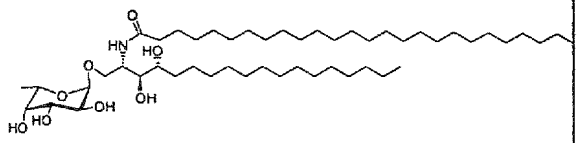
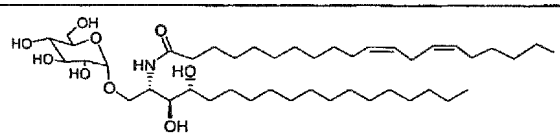
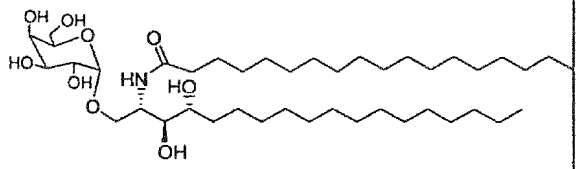
Several of these compounds also showed activity at modulating cytokines in mice (see, examples). Both DB03-4 and DB03-5 show a bias towards inducing Type 2 cytokines, *i.e.*, cytokines that have anti-inflammatory effect, and are strong inducers of IL-4 in iNKT cells with blunted IFNγ and NK cell transactivation. DB03-3 is a good inducer of IL-4 and, in some murine strains, a strong inducer of IFNγ in vitro. High level production of IFNγ is often associated with NK cell activation. DB03-9, and DB03-10 are strong inducers of IL-4, weak inducers of IL-2, and moderate inducers of IFNγ in vitro. YTC03-17 shows strong agonist activity in in vitro studies. DB03-8 is a weak agonist of iNKT cells in vitro and exacerbates SLE in NZB/W-F1 mice. It is believed that DB03-8 may be a possible antagonist/partial agonist, *i.e.*, it inhibits the direct and indirect activity of iNKT because it acts as an antagonist of iNKT or because it stimulates an abortive partial activation.

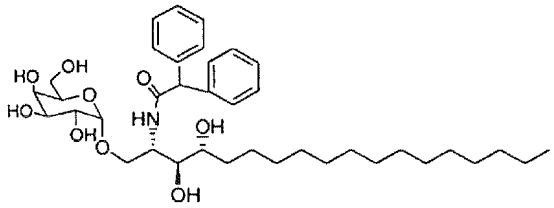
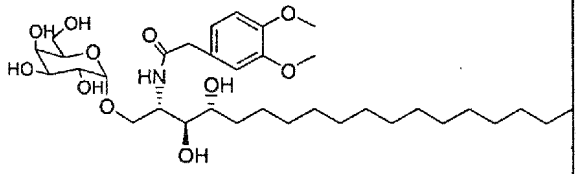
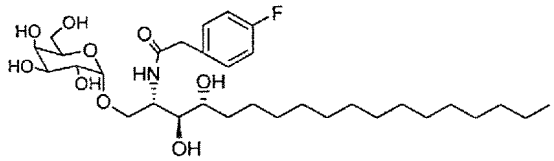
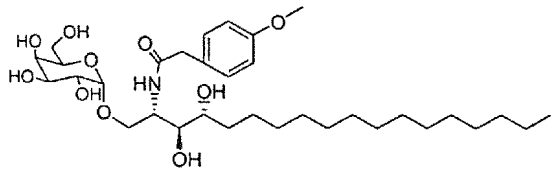
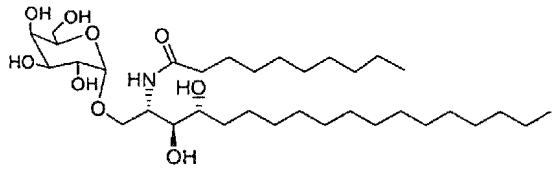
The term iNKT cells, as used herein, mean the specific subset of CD1d-dependent T cells that expresses the invariant TCRα chain rearrangement consisting of Vα14-Jα18 in mice, and Vα24-Jα18 in humans. These cells are uniformly reactive to α-galactosylceramides presented by CD1d. These cells are also referred to as "Type 1" NKT cells, and the distinction between the different types of NKT cells and the nomenclature relating to this are summarized in the publication by Godfrey et al., Nat Rev Immunol. 2004 Mar;4(3):231-7. Methods for determining cytokine production by iNKT cells in response to CD1d-presented glycolipids for determination of strong or weak agonist activity are described in Yu KOA, Im JS, Molano A, Dutronc Y, Illarionov PA, Forestier C, Fujiwara N, Arias I, Miyake S, Yamamura T, Chang Y-T, Besra GS, Porcelli SA, Modulation of CD1d-restricted NKT cell responses using N-acyl variants of α-galactosylceramides, Proc. Nat. Acad. Sci. (USA), 102:3383-8 (2005), see also Godfrey DI et al., *Nat Rev Immunol.* 2004 Mar;4(3):231-7.

YTCO3-24 and YTCO3-25 show enhanced IL-4 induction relative to IFNγ in vitro with splenocytes from NZB/W F1 mice. DB03-6 is an agonist in splenocyte cultures with apparent enhancement of IL-4 relative to IFNγ and minimal IL-2 induction.

Additional representative ceramide-like glycolipids of the invention include those provided in the table below:

Compound	Monosaccharide	Molecular Weight	Structure
DB04-01 (KRN7000)[a]	α-D-Gal	858.32	
DB01-1	α-D-Gal	830.27	
DB02-1	α-D-Glu	830.27	
DB03-4	α-D-Gal	770.13	
DB03-5	α-D-Gal	766.10	
DB03-8	α-L-Fuc	814.27	
DB04-9	β-D-Man	770.13	
DB05-9	α-D-Gal	742.08	
DB05-10	α-D-Gal	740.06	

DB05-11	α-D-Gal	742.08	
DB05-12	α-D-Gal	770.13	
DB05-14	α-D-Gal	740.06	
DB05-15	α-D-Gal	742.08	
DB05-16	α-D-Gal	742.08	
DB05-17	α-D-Gal	740.06	
DB06-14	*α*-L-Fuc	842.32	
DB06-15	*α*-D-Glu	770.13	
YTC03-15	α-D-Gal	746.11	

YTC03-17	α-D-Gal	705.96	
YTC03-24	α-D-Gal	657.83	
YTC03-30	α-D-Gal	615.77	
YTC03-33	α-D-Gal	627.81	
YTC03-34	α-D-Gal	633.90	

DB04-01 (KRN7000) is not a compound of the invention.

The following synthetic scheme depicts a synthetic methodology used to make the ceramide-like glycolipids:

5

expressing CD1d, or in the presence of recombinant CD1d proteins bound to the tissue culture plate surface (FIG. 5).

The ability of the compounds to activate NK T cell hybridomas in the presence of various types of cells expressing CD1d reported in FIG. 5A was determined using a murine NK T cell hybridoma stimulation assay. CD1d-transfected RMA-S cells were plated at 50,000 cells/well in flat-bottom tissue culture plates in 100 microliters complete medium containing varying concentrations of ceramide-like glycolipids for 6 h at 37°C. The plates were then centrifuged (430g, 3 min), and cells washed with PBS three times. Fifty thousand NKT hybridoma cells (clone DN3A4.1-2) were then added in 100 microliters of medium for a 12 h stimulation. Cell-free supernatants were collected at the end of incubation and assayed for IL-2 by standard capture ELISA. Relative potencies of the ceramide-like glycolipids were calculated from the reciprocal effective concentrations at half-maximal response ($1/EC_{50}$), and expressed as units by normalization to the observed potency for KRN7000. The structure of the ceramide-like glycolipids are provided in FIG. 5B.

The results of these screening assays revealed that between 5-10% of the analogues in our collection had potency equal to or greater than that of KRN7000 (potency was defined by the molar concentration required to give a half maximal response of the NK T cell hybridomas). In the representative assay shown in FIG. 5A, seven compounds showed substantially greater potency than KRN7000. In addition, several of these compounds with elevated potency also showed increased maximal activity when compared to KRN7000 (defined as the highest level of response achieved in the assay over the range of compound dilutions tested; data not shown).

Table 1 shows results from the proliferation assay, IL-4 assay, and IFNγ assay for compounds YTCO3-42 to YTCO3-61, showing variability in their ability to induce proliferation and induce the two cytokines, as well as the ratio of IL-4 to IFNγ. FIG. 5C shows a graphical representation of the results reported in Table 1 for cytokine response. Table I and FIG. 5C shows that cytokine production by normal mouse splenocytes is stimulated with ceramide-like glycolipids. The cytokine response reported in Table 1 and FIG. 5C was determined by the following procedure: Bulk splenocytes from C57BL/6 mice were plated at 300,000 per well in 96-well flat-bottom tissue culture plates with ceramide-like glycolipids diluted to a concentration of 500 nM in 200 microliters complete medium. After 48 h at 37°C, 150 microliters of supernatant was removed for cytokine measurements. Supernatant levels of IL-4, and IFNγ were measured by standard enzyme-linked immunosorbent assay (ELISA), using capture and biotinylated detection antibody pairs

(clones 11B11 / BVD6-24G2-biotin, and R4-6A2 / XMG1.2-biotin, respectively, from BD Pharmingen, San Diego, California). Cytokine content was revealed using streptavidin-horseradish peroxidase (Zymed, South San Francisco, California) with TMB-Turbo substrate (Pierce, Rockford, Illinois) or alternatively with streptavidin-alkaline phosphatase (Zymed) with 4-nitrophenyl phosphate disodium hexahydrate substrate (Sigma-Aldrich, St. Louis, Missouri) and read at 450 or 405 nm, respectively, on a microplate reader (Titertek, Huntsville, Alabama). Standards for IL-4 and IFNγ were from Peprotech (Rocky Hill, New Jersey).

Table 1 shows stimulation of CD1d-dependent proliferation by compounds YTCO3-42 to YTCO3-61. The results were obtained using a splenocyte proliferation assay according to the following procedure: Bulk splenocytes from C57BL/6 mice were plated at 300,000 per well in 96-well flat-bottom tissue culture plates with the indicated ceramide-like glycolipid diluted in complete medium to the concentrations shown. After 48 h at 37°C, the plate wells were pulsed with 20 microliters of medium containing 50 microCi/ml ^{3}H-thymidine and incubated for a further 18 h. Cell proliferation was estimated by harvesting pulsed cells onto 96-well filtermats and scintillation counting on a 1450 Microbeta Trilux instrument (Wallac/Perkin Elmer, Boston, Massachusetts). FIG. 5D shows stimulation of CD1d-dependent proliferation by DB02-1, an α-glucosyl ceramide identical to DB01-1 except that a glucose replaces the galactose of DB01-1. The results reported in FIG 5D were obtained by the same method used to obtain the data for proliferation provided in Table 1.

Table 1. EC_{50} is the concentration of glycolipid that gave a 50% maximal response in proliferation of splenocyte cultures as measured by ^{3}H-thymidine incorporation. Higher EC_{50} values thus represent lower potency for stimulation of NKT cell dependent proliferation. IL-4 and IFNγ levels were measured by ELISA in culture supernatants of splenocyte cultures stimulated for 48 hours with 500 nM of each glycolipid. Cytokine concentrations are in ng/ml. ND indicates below the reliable level for detection by the assay. The values in parentheses are the IL-4/IFNγ ratios for each compound divided by this ratio for the KRN7000-like DB01-1 compound.

COMPOUND	Proliferation EC_{50} (nM)	Cytokine Response		
		IL-4	IFNγ	ratio IL-4/IFNγ
DBO1-1	63.6	0.19	7.29	0.026 (1.0)
YTCO3-42	>2500	ND	ND	-
YTCO3-43	86.9	0.35	19.50	0.018 (0.7)
YTCO3-44	57.1	1.38	14.19	0.097 (3.7)
YTCO3-45	222.3	0.63	15.26	0.041 (1.6)
YTCO3-46	4.8	0.22	8.52	0.026 (1.0)
YTCO3-47	7.5	0.21	19.63	0.011 (0.4)
YTCO3-48	86.9	0.20	12.56	0.016 (0.6)
YTCO3-49	57.1	0.13	4.57	0.028 (1.1)
YTCO3-50	5.4	0.07	9.47	0.007 (0.3)
YTCO3-51	63.8	0.05	9.03	0.006 (0.2)
YTCO3-52	17.0	0.19	10.25	0.019 (0.7)
YTCO3-53	38.0	0.02	0.67	0.030 (1.2)
YTCO3-54	17.2	0.11	10.39	0.011 (0.4)
YTCO3-55	675.0	ND	ND	-
YTCO3-56	11.4	0.22	11.79	0.019 (0.7)
YTCO3-57	2188.9	ND	ND	-
YTCO3-58	51.8	0.21	8.64	0.024 (0.9)
YTCO3-59	0.1	1.77	0.22	8.045 (309.4)
YTCO3-60	10.9	0.18	8.18	0.022 (0.8)
YTCO3-61	72.4	0.17	7.50	0.023 (0.9)

Structure/activity relationships of the analogues. The compounds of greatest interest to emerge from the screening assays fall mainly into two general categories. They are either α-galactosylceramides containing truncated fatty acyl chains with various degrees of unsaturations, or α-galactosylceramides with aromatic rings in their amide linked branch. Our results indicate that these alterations in the amino substitution of KRN7000 can have at least three possible effects on the biological activity of the compounds: 1) change in potency/activity, 2) change in the type of cell that presents the compound efficiently, and 3) change in the outcome of NK T cell activation in terms of the types of cytokines produced. Illustrative examples of these effects are provided by the figures that follow.

FIG. 6 shows the differential presentation of various potent ceramide-like glycolipids when different types of antigen presenting cells (APCs) are used in the NK T cell activation assay. IL-2 production by NK T hybridoma DN32D3 in response to eight selected ceramide-like glycolipids, was determined using three different cell types as antigen presenting cells. RMA-S.mCD1d are a mouse lymphoma line that has been transfected to express murine CD1d. JAWS II is a mouse dendritic cell line that naturally expresses mouse CD1d. HeLa.hCD1d is a human cervical carcinoma cell line that has been transfected to express human CD1d. The assay was performed in the same manner as described above for the results depicted in Figure 5A.

Note, for example, that YTC03-17 is markedly more potent than KRN7000 when the compounds are presented by CD1d molecules expressed on a lymphoid cell line (RMA-S), whereas these two compounds are presented about equally by CD1d molecules expressed on a epithelial tumor cell line (HeLa). When a dendritic cell line (JAWS-II) is used as the antigen presenting cell, there is very little or no response at all to YTC03-17. In addition to demonstrating that a biphenyl substitution can generate an active compound, these studies show that the activity is markedly dependent on the type of cell which bears the CD1d on which the compound is presented.

FIG. 6 also shows additional phenyl containing analogues that displayed enhanced potency in some assays. Again the results are quite dependent on the type of antigen presenting cell used. Note for example that YTC03-30 has extraordinary potency when presented by HeLa cells (at least 100 fold greater than KRN7000), but similar potency to KRN7000 when presented by RMA-S cells.

FIG. 7 shows effects of varying the length of the N-linked fatty acid when the fatty acid chain is fully saturated. Note that there appears to be a clear influence of chain length on potency, with optimal activity occurring at a length of C12 in this particular system. How this effect will be influenced by changing the nature of the antigen-presenting

cell remains to be investigated. Figure 8 shows the effects of introducing unsaturations into the fatty acid chain when the fatty acid length is held constant at C20. A dramatic effect on potency is observed, with a diunsaturated analogue (DB03-4) having extremely enhanced potency. Again, how this effect will be influenced by changing the nature of the antigen-presenting cell remains to be investigated. The relative potency of each analogue was determined by measuring IL-2 production by mouse NK T cell hybridoma DN3A4.1-2 as described above for the results depicted in FIG. 5A.

FIG. 8 shows the effects of fatty acid chain unsaturations on potency of C20 fatty acid ceramide-like glycolipids. The ceramide-like glycolipids with the indicated chain lengths and indicated numbers of double bonds were tested for stimulation of NK T hybridoma DN32.D3 by measuring IL-2 production by mouse NK T cell hybridoma DN3A4.1-2 as described above for the results depicted in FIG. 7.

A most intriguing property of KRN7000 analogues is that in some cases they may elicit immune responses that are qualitatively different from those that occur following stimulation with the parent compound. This was shown to be the case with the sphingosine chain length variant OCH, as published by Yamamura and colleagues (Miyamoto et al., 2001). In that case, it was shown that OCH elicited a selective production of interleukin-4 (IL-4) when administered *in vivo* to mice, and failed to stimulate the strong production of interferon-γ (IFNγ) that is observed after injection of KRN7000. This selective activation of IL-4 production by NK T cells was proposed to be the basis for the enhanced therapeutic effects of OCH in the EAE model of central nervous system autoimmune disease. We have observed a similar *in vivo* effect on the nature of the NK T cell response using several of our ceramide-like glycolipids. As shown in FIG. 9, two of the ceramide-like glycolipids containing unsaturated C20 fatty acids (DB03-4 and DB03-5) elicit a strong IL-4 response two hours after injection into mice. These responses are similar to those seen for DB01-1, an analogue that is structurally almost identical to KRN7000 (C24 fatty acid instead of C26, otherwise identical) and indistinguishable in terms of its activity in our hands. However, while DB01-1 also evokes a strong IFNγ response at 20 hours post injection, this late wave of IFNγ is not seen with the two C20 ceramide-like glycolipids. This selective IL-4 induction is virtually identical to that reported for the OCH analogue, and thus illustrates the potential for amino substituted analogues of KRN7000 to induce qualitatively different immunomodulatory effects *in vivo*. The results reported in FIG. 9 were obtained by measuring serum levels of IL-4 and IFNγ after administering a single injection of DB01-1, DB03-4, or DB03-5 to C57BL/6 mice. C57BL/6 mice (11-13 weeks old) were given a single i.p. injection of 4.8 nanomoles of the ceramide-like glycolipids or PBS/vehicle

control. Serum cytokine levels were measured 2 and 20 hours later by capture ELISA. Bars show means of three mice, with standard deviation.

FIG. 11, shows the differential effect of various KRN7000 analogs in stimulating CD40L expression. FIG. 11 also shows that the galactose moiety of these ceramide compounds can be replaced with another monosaccharide while still retaining some activity, since DB03-8, which has a fucose replacing the galactose, was capable of inducing CD40L. The upregulation of CD40L by ceramide-like glycolipids reported in FIG. 11 were obtained by incubating NK T hybridoma DN3A4.1-2 with RMA-S/CD1d cells at a ratio of 2:1 in the presence of 0.5 uM of ceramide-like glycolipid for 18 hours. Cells were then resuspended and labeled with mAbs specific for CD5 and CD40L. Levels of CD40L were determined by FACS analysis of the population gated for CD5 staining.

See also FIG. 5D, showing stimulation of CD1d-dependent proliferation by the α-glucosylceramide DB02-1. DB02-1 also stimulated significant cytokine production, including both IFNγ and IL-4. Interestingly, while IFNγ levels produced in response to DB02-1 in *in vitro* splenocyte cultures were markedly lower than those stimulated by DB01-1 at all concentrations of the analogs, the IL-4 levels were nearly equivalent at doses of 100 μM or greater. This suggests that DB02-1 is an NKT cell agonist with the potential to stimulate a TH2-biased cytokine response.

Given the widely held belief that selective augmentation of IL-4 production can be protective or therapeutic in the setting of many autoimmune diseases, we have initiated studies to examine the efficacy of compounds such as DB03-4 and DB03-5 in diabetes prone NOD mice. This work indicates that our ceramide-like glycolipids are superior to KRN7000 and the KRN7000-mimic DB01-1 in the prevention of diabetes in NOD mice (FIG. 10). FIG. 10 shows that type 1 diabetes can be delayed or prevented in NOD mice treated with ceramide-like glycolipids. The results reported in FIG. 10 were obtained by treating three groups, each consisting of 9-12 female NOD mice, starting from age 5 weeks with a ceramide-like glycolipid. The indicated ceramide-like glycolipids (DB03-4, DB03-5, or KRN7000) were injected i.p. once per week in a dose of 200 micrograms/kg. Treatment was discontinued at 11 weeks of age, and the mice were monitored weekly for glucosuria (top) and death (bottom).

For experiments involving in vivo treatment of mice with the ceramide-like glycolipids, the ceramide-like glycolipids were administered by i.p. or i.v. injection in 0.2 ml PBS + 0.025% Tween-20, or in vehicle alone. A typical dose is about 4-5 nmoles per animal per injection. Representative references for administering an α-galactosyl ceramide to mice by i.p., i.v. or p.o. routes are S. Sharif et al., *Activation of natural killer T cells by*

alpha-galactosylceramide treatment prevents the onset and recurrence of autoimmune Type 1 diabetes, Nat Med., Sep. 7, 2001, (9):1057-62 and K. Miyamoto et al., *A synthetic glycolipid prevents autoimmune encephalomyelitis by inducing TH2 bias of natural killer T cells*, Nature. Oct. 4, 2001, 413(6855):531-4.

Compounds DB04-1 (KRN-7000), DB05-9, DB05-10, DB05-11, DB05-12, DB05-14, DB05-15, DB05-16, and DB05-17 were also tested for their ability to induce proliferation and to induce the cytokines IFNγ and IL-4. Each of these compounds of the invention has conjugated double bonds in R1. The ability to induce the cytokines IFNγ and IL-4 was determined using the same IFNγ assay and IL-4 assay as described above for compounds YTC03-42 to YTC03-61. The ability of these compounds to induce proliferation was determined using the same splenocyte proliferation assay described above for compounds YTC03-42 to YTC03-61.

FIG. 12 shows results from the IFNγ assay and FIG. 13 shows the results from IL-4 assay for compounds DB04-1 (KRN-7000), DB05-9, DB05-10, DB05-11, DB05-12, DB05-14, DB05-15, DB05-16, and DB05-17. FIG. 14 shows stimulation of CD1d-dependent proliferation by each of these compounds, determined as described above.

FIG. 15 shows maximum IFNγ production and maximum IL-4 production when cells are treated with 500 nM of DB04-1 (KRN-7000), DB05-9, DB05-10, DB05-11, DB05-12, DB05-14, DB05-15, DB05-16, or DB05-17 and FIG. 16 shows the ratio of IL-4 production to IFNγ production for each of these compounds.

These results demonstrate that these compounds of the invention, with conjugate double bonds, can stimulate greater proliferative and functional activity of NKT cells than KRN-7000. Also, in some cases the compounds show a bias for high secretion levels of IL-4 relative to IFNγ.

These studies have identified a panel of novel immunologically active analogues of αGalCer. These compounds differ significantly in structure from the previously studied and well-documented prototype in this family, KRN7000. We have already demonstrated a number of important properties for certain analogues that would make them superior agents for a variety of applications in the prevention and treatment of disease. These compounds are also useful as adjuvants for stimulation of responses to vaccines, for immunotherapy against allergic diseases, and for the treatment of cancer.

In view of the above, it will be seen that the several advantages of the invention are achieved and other advantages attained.

As various changes could be made in the above methods and compositions without departing from the scope of the invention, it is intended that all matter contained in the

FIG. 5A

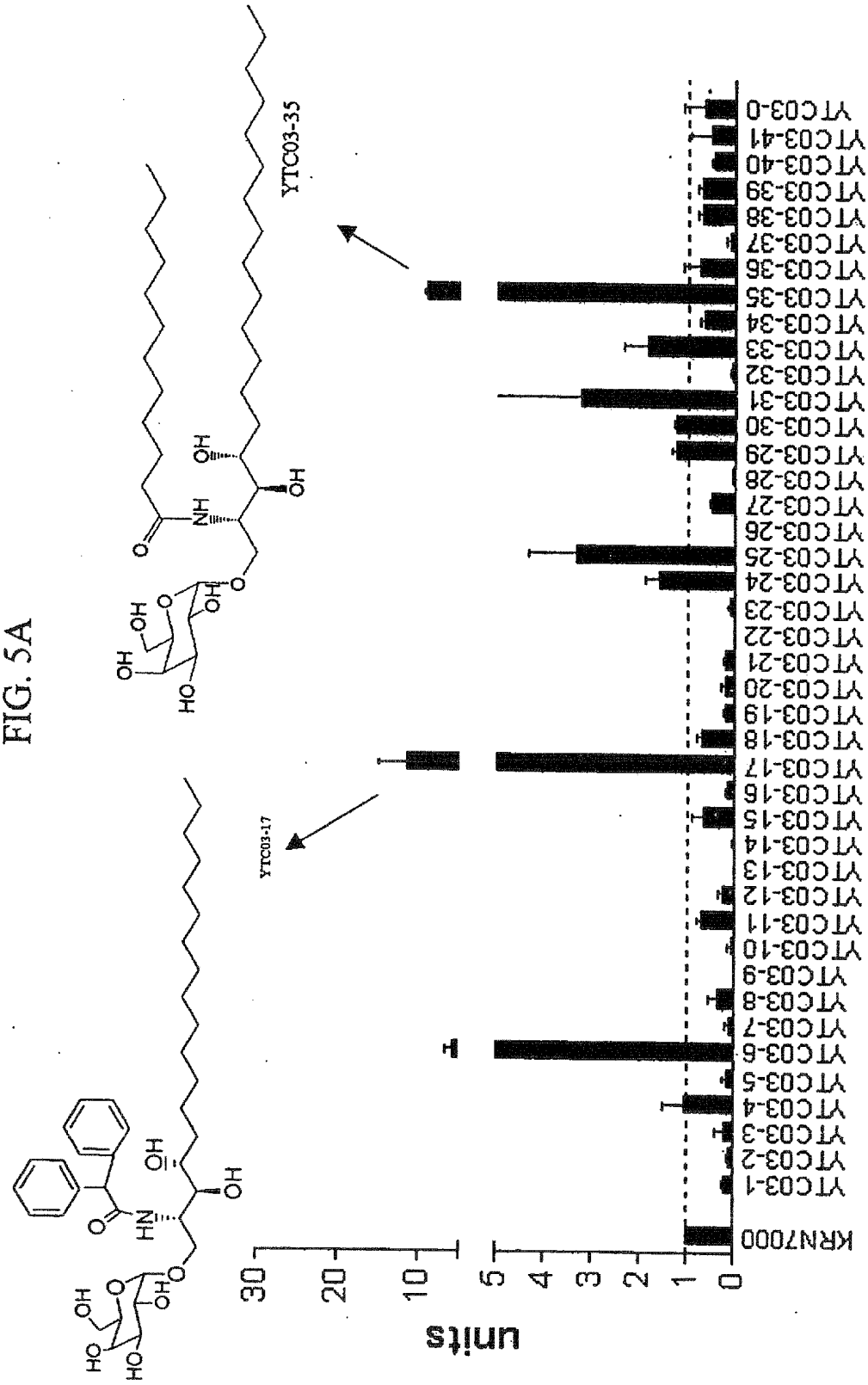

YTC03-17
YTC03-35
units
0
1
2
3
4
5
10
20
30
KRN7000
YTC03-1
YTC03-2
YTC03-3
YTC03-4
YTC03-5
YTC03-6
YTC03-7
YTC03-8
YTC03-9
YTC03-10
YTC03-11
YTC03-12
YTC03-13
YTC03-14
YTC03-15
YTC03-16
YTC03-17
YTC03-18
YTC03-19
YTC03-20
YTC03-21
YTC03-22
YTC03-23
YTC03-24
YTC03-25
YTC03-26
YTC03-27
YTC03-28
YTC03-29
YTC03-30
YTC03-31
YTC03-32
YTC03-33
YTC03-34
YTC03-35
YTC03-36
YTC03-37
YTC03-38
YTC03-39
YTC03-40
YTC03-41
YTC03-0

FIG. 5C

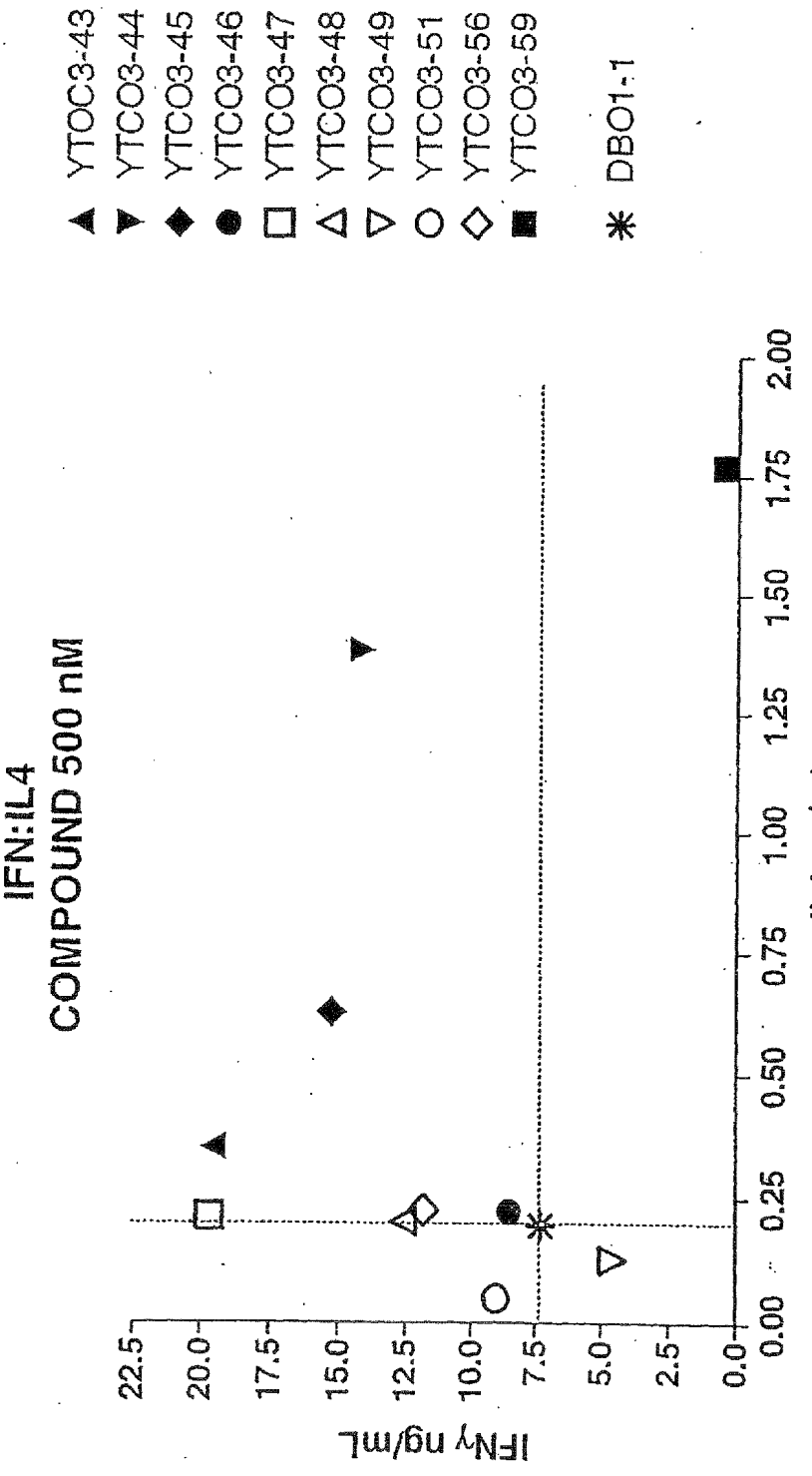

IFN:IL4
COMPOUND 500 nM
YTOC3-43
YTCO3-44
YTCO3-45
YTCO3-46
YTCO3-47
YTCO3-48
YTCO3-49
YTCO3-51
YTCO3-56
YTCO3-59
DBO1-1
IFNγ ng/mL
IL4 ng/mL
0.0
2.5
5.0
7.5
10.0
12.5
15.0
17.5
20.0
22.5
0.00
0.25
0.50
0.75
1.00
1.25
1.50
1.75
2.00

FIG. 5D

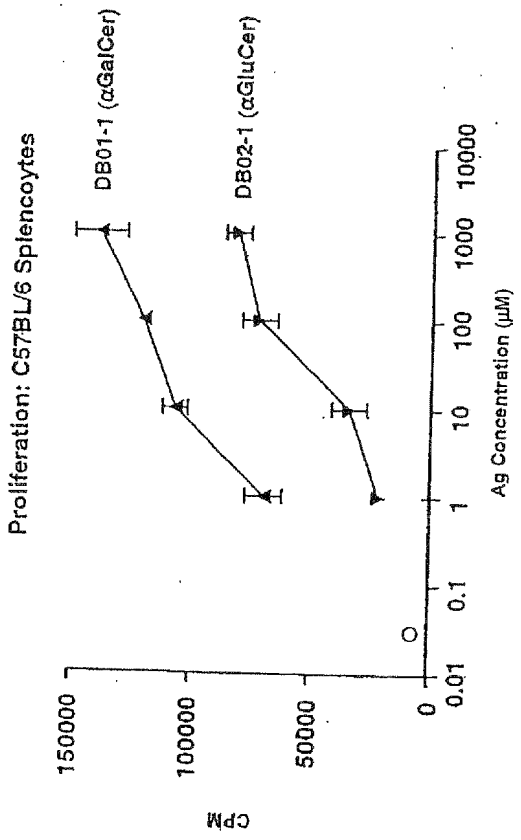

Proliferation: C57BL/6 Splencoytes
DB01-1 (αGalCer)
DB02-1 (αGluCer)
CPM
150000
100000
50000
0
0
0.01
0.1
1
10
100
1000
10000
Ag Concentration (μM)

FIG. 6

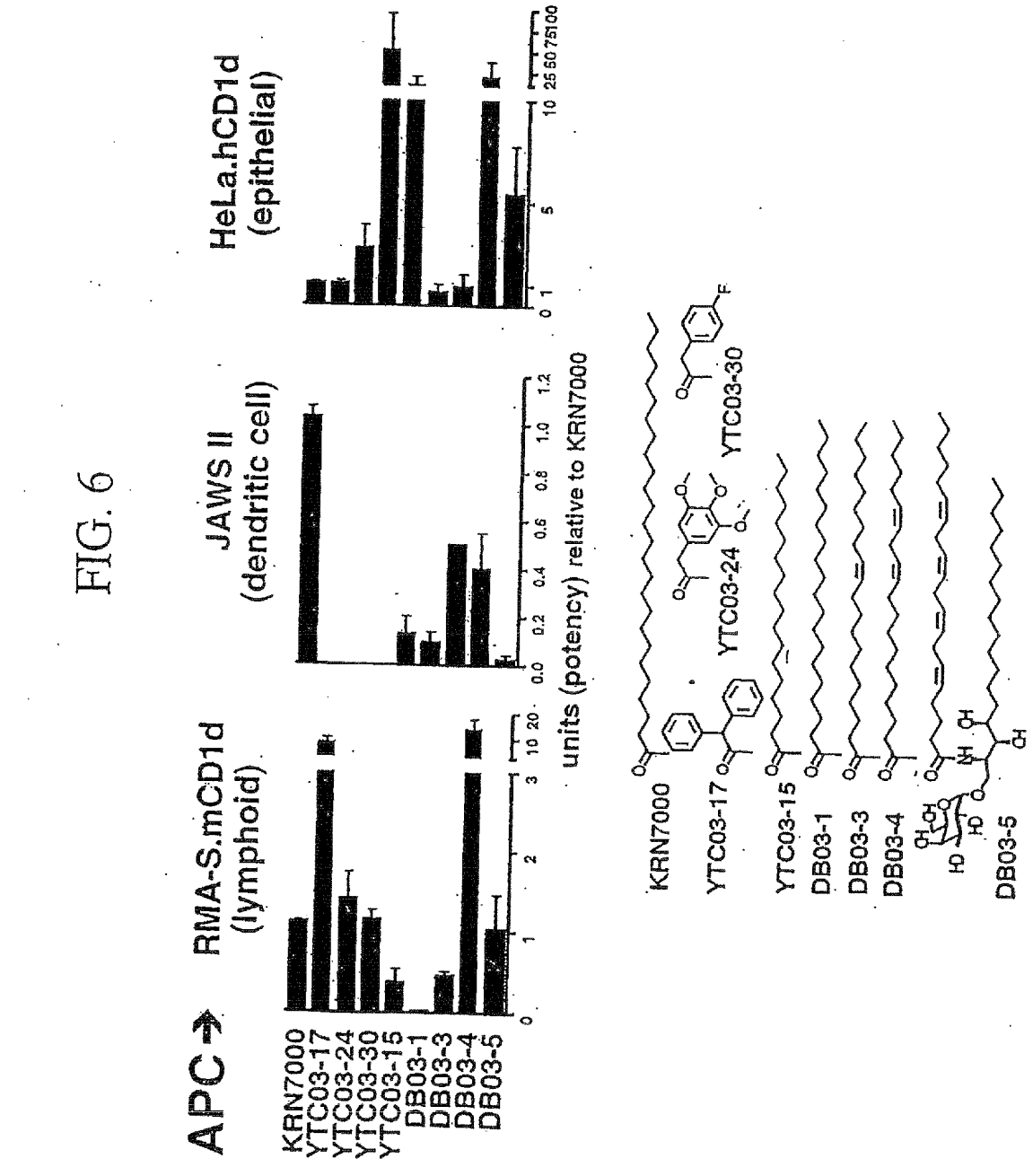

APC →
RMA-S.mCD1d (lymphoid)
JAWS II (dendritic cell)
HeLa.hCD1d (epithelial)
KRN7000
YTC03-17
YTC03-24
YTC03-30
YTC03-15
DB03-1
DB03-3
DB03-4
DB03-5
units (potency) relative to KRN7000
0 1 2 3 10 20
0.0 0.2 0.4 0.6 0.8 1.0 1.2
0 1 5 10 25 50 75 100
KRN7000
YTC03-17
YTC03-24
YTC03-30
YTC03-15
DB03-1
DB03-3
DB03-4
DB03-5

FIG. 7

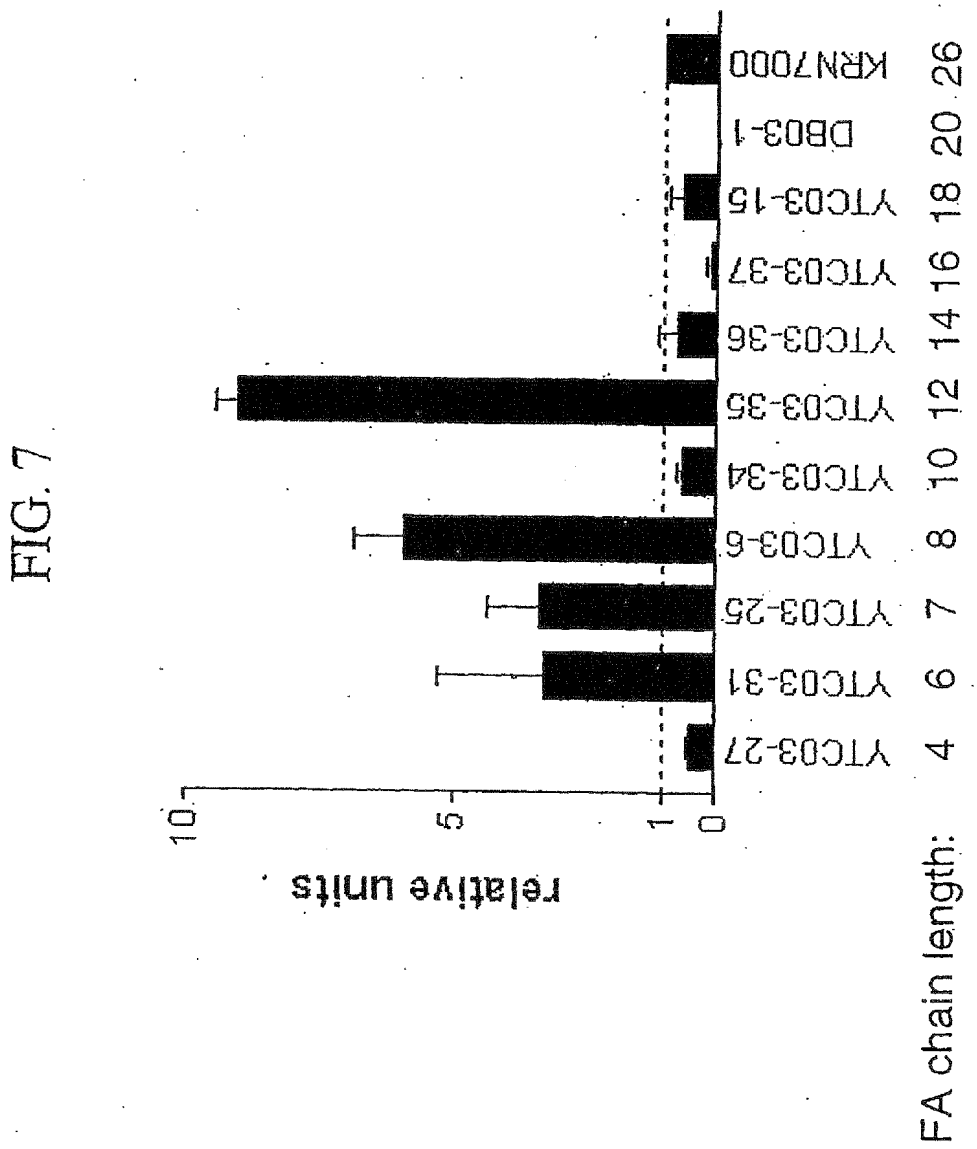

relative units
10
5
1
0
YTC03-27
YTC03-31
YTC03-25
YTC03-6
YTC03-34
YTC03-35
YTC03-36
YTC03-37
YTC03-15
DB03-1
KRN7000
FA chain length: 4 6 7 8 10 12 14 16 18 20 26

FIG. 8

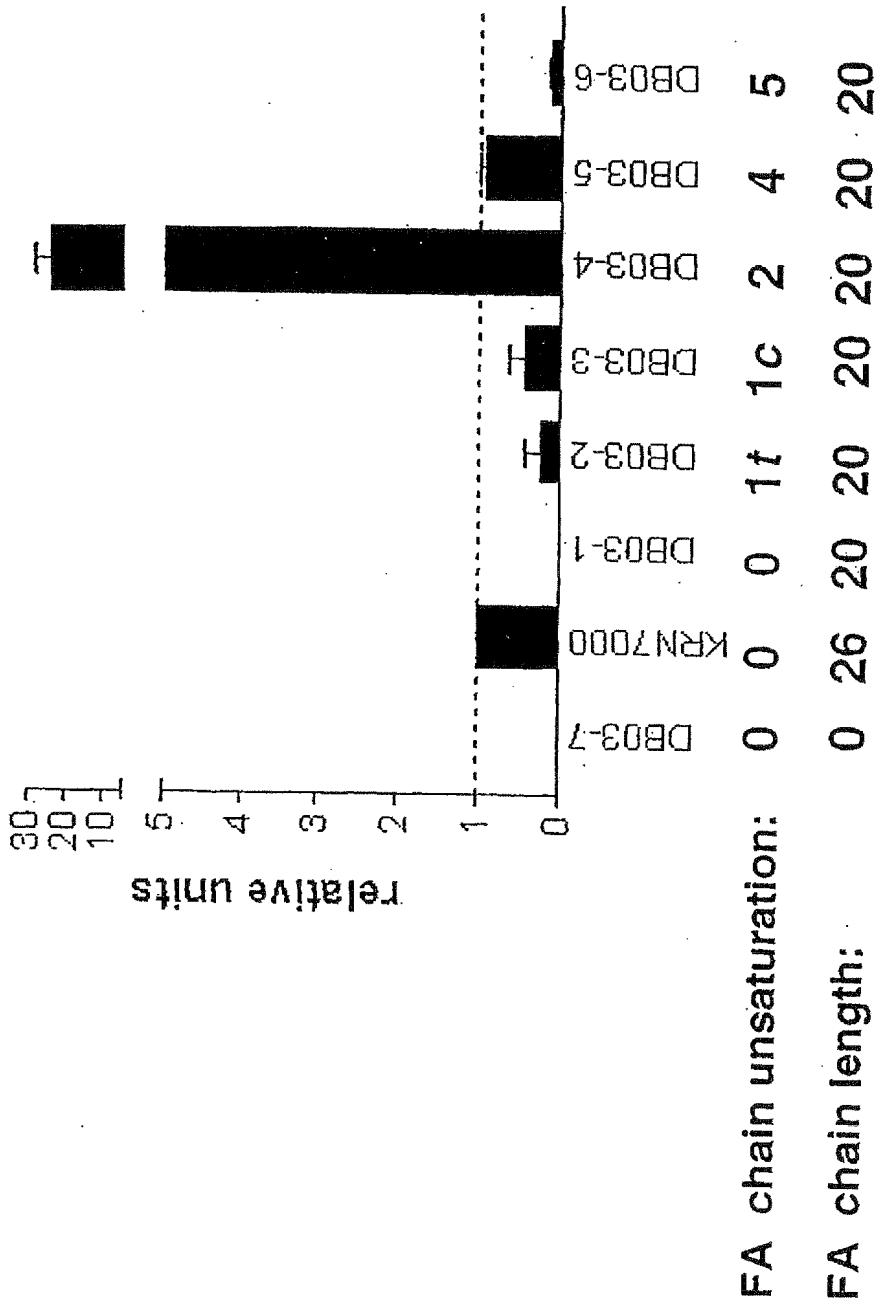

relative units
30
20
10
5
4
3
2
1
0
DB03-7
KRN7000
DB03-1
DB03-2
DB03-3
DB03-4
DB03-5
DB03-6
FA chain unsaturation: 0 0 0 1t 1c 2 4 5
FA chain length: 0 26 20 20 20 20 20 20

FIG. 9

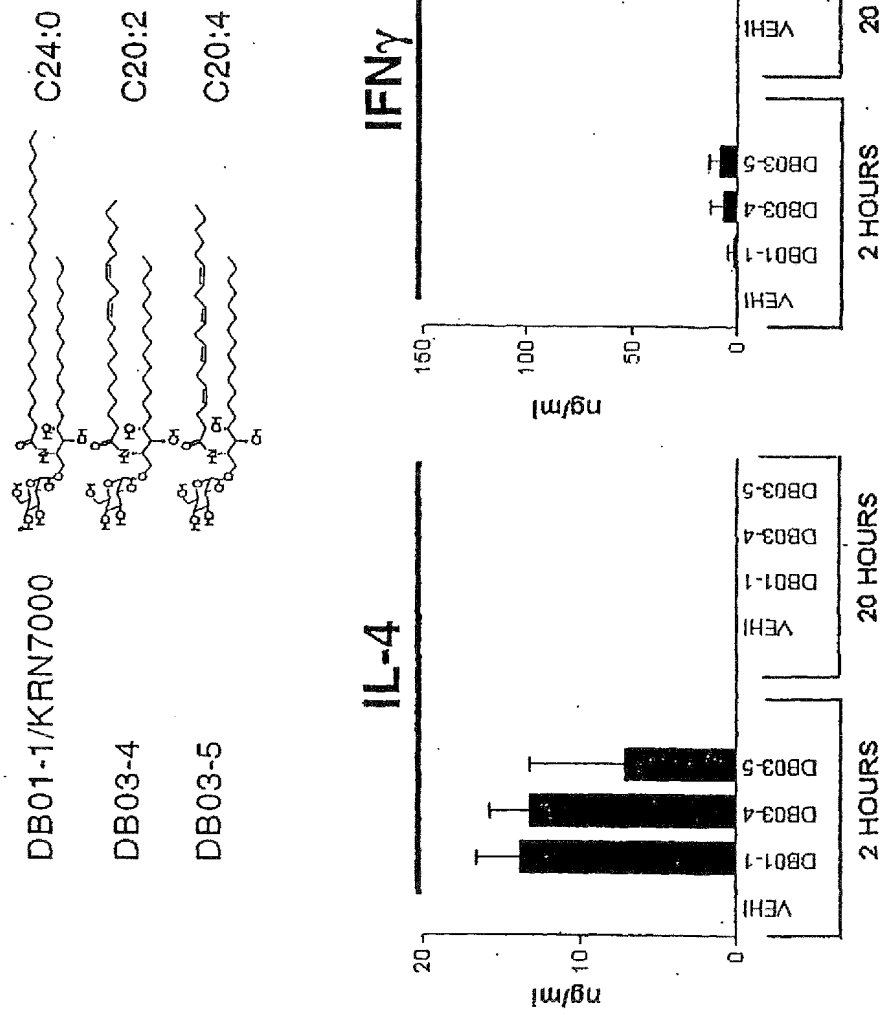
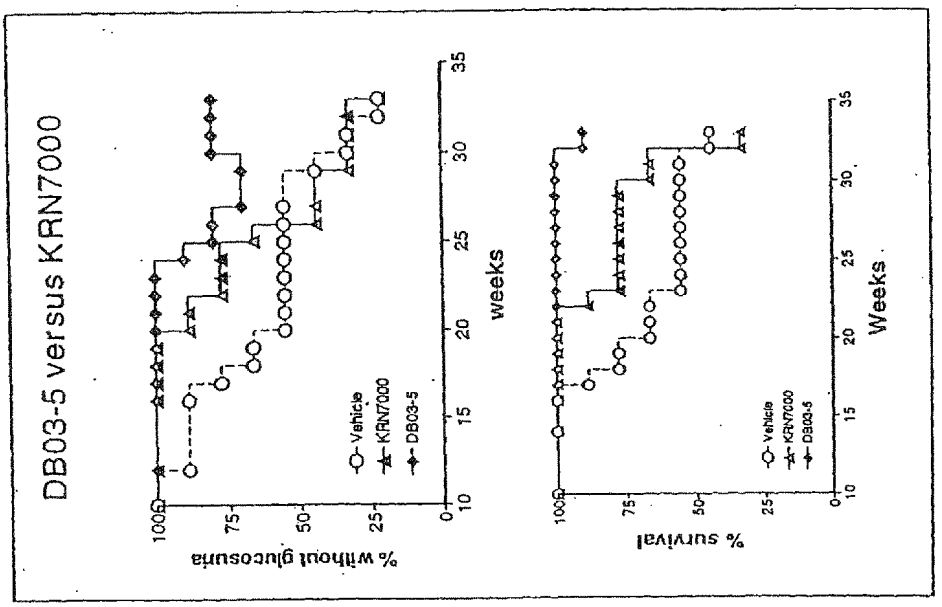

DB01-1/KRN7000
DB03-4
DB03-5
C24:0
C20:2
C20:4
IL-4
IFNγ
ng/ml
VEHI
DB01-1
DB03-4
DB03-5
2 HOURS
20 HOURS

FIG. 10


DB03-5 versus KRN7000
% without glucosuria
% survival
weeks
Weeks
Vehicle
KRN7000
DB03-5
100
75
50
25
0
10
15
20
25
30
35

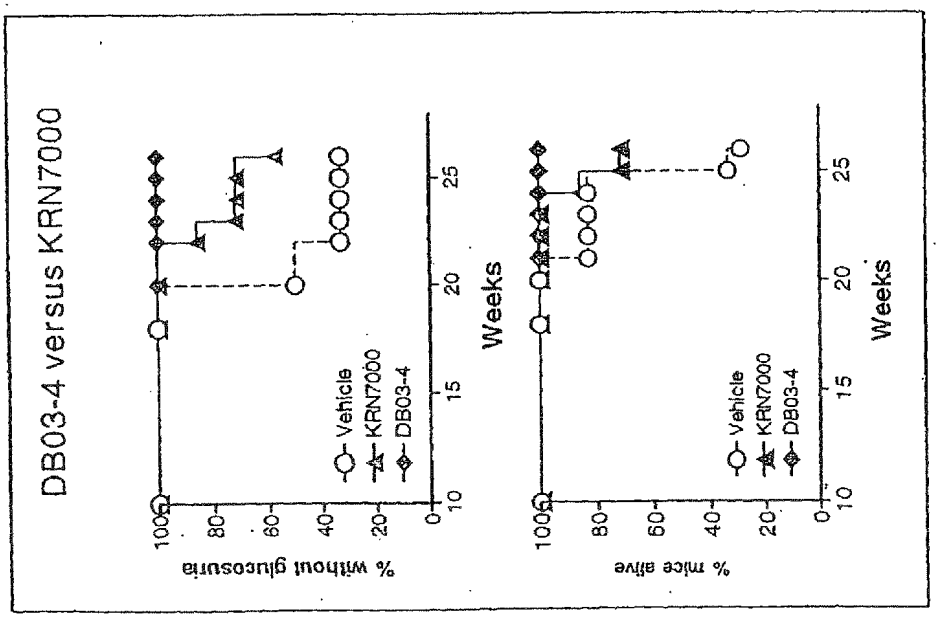

DB03-4 versus KRN7000
% without glucosuria
% mice alive
Weeks
Vehicle
KRN7000
DB03-4
100
80
60
40
20
0
10
15
20
25

FIG. 11

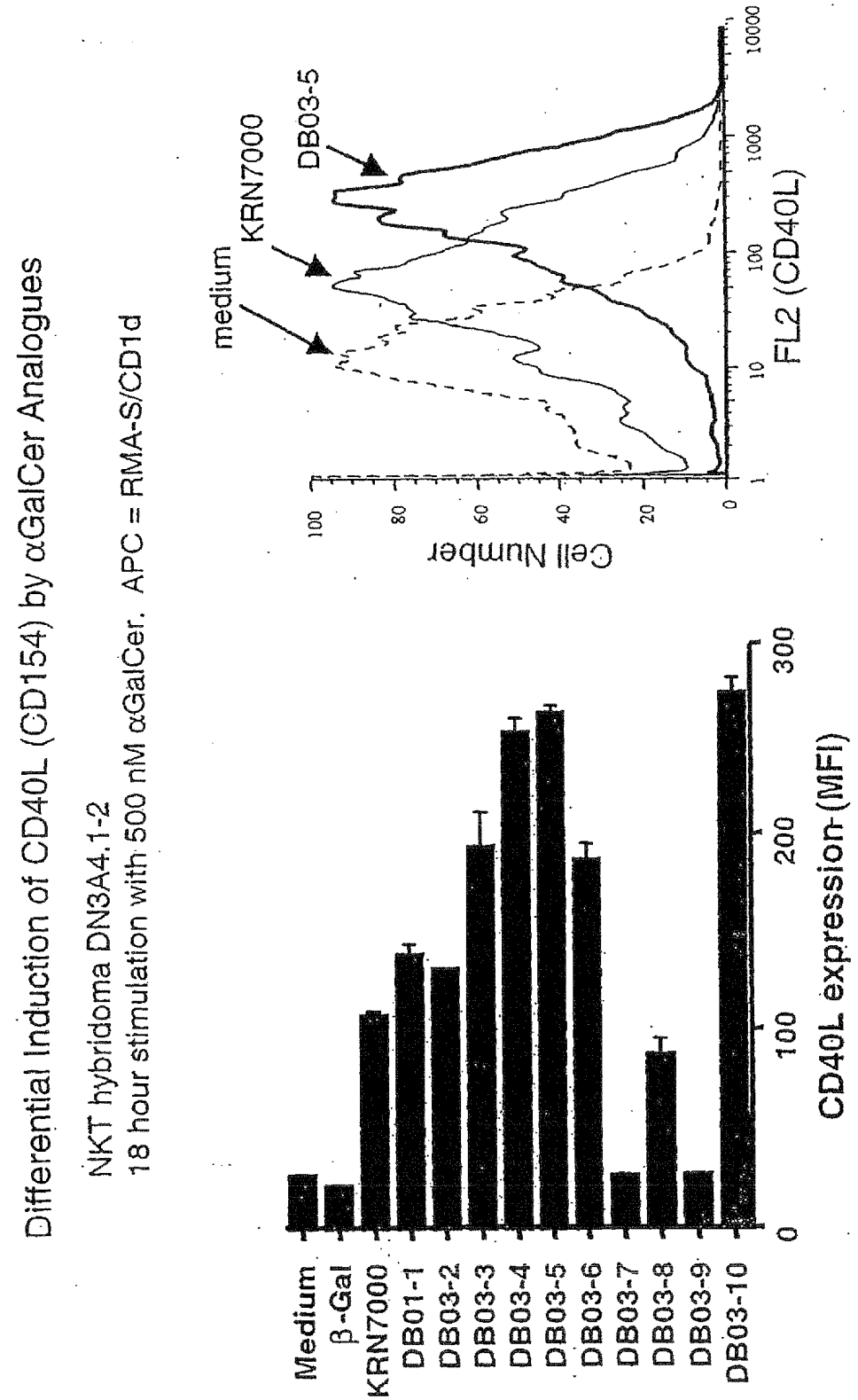

Differential Induction of CD40L (CD154) by αGalCer Analogues
NKT hybridoma DN3A4.1-2
18 hour stimulation with 500 nM αGalCer. APC = RMA-S/CD1d
Medium
β-Gal
KRN7000
DB01-1
DB03-2
DB03-3
DB03-4
DB03-5
DB03-6
DB03-7
DB03-8
DB03-9
DB03-10
0
100
200
300
CD40L expression (MFI)
medium
KRN7000
DB03-5
Cell Number
FL2 (CD40L)

FIG. 12

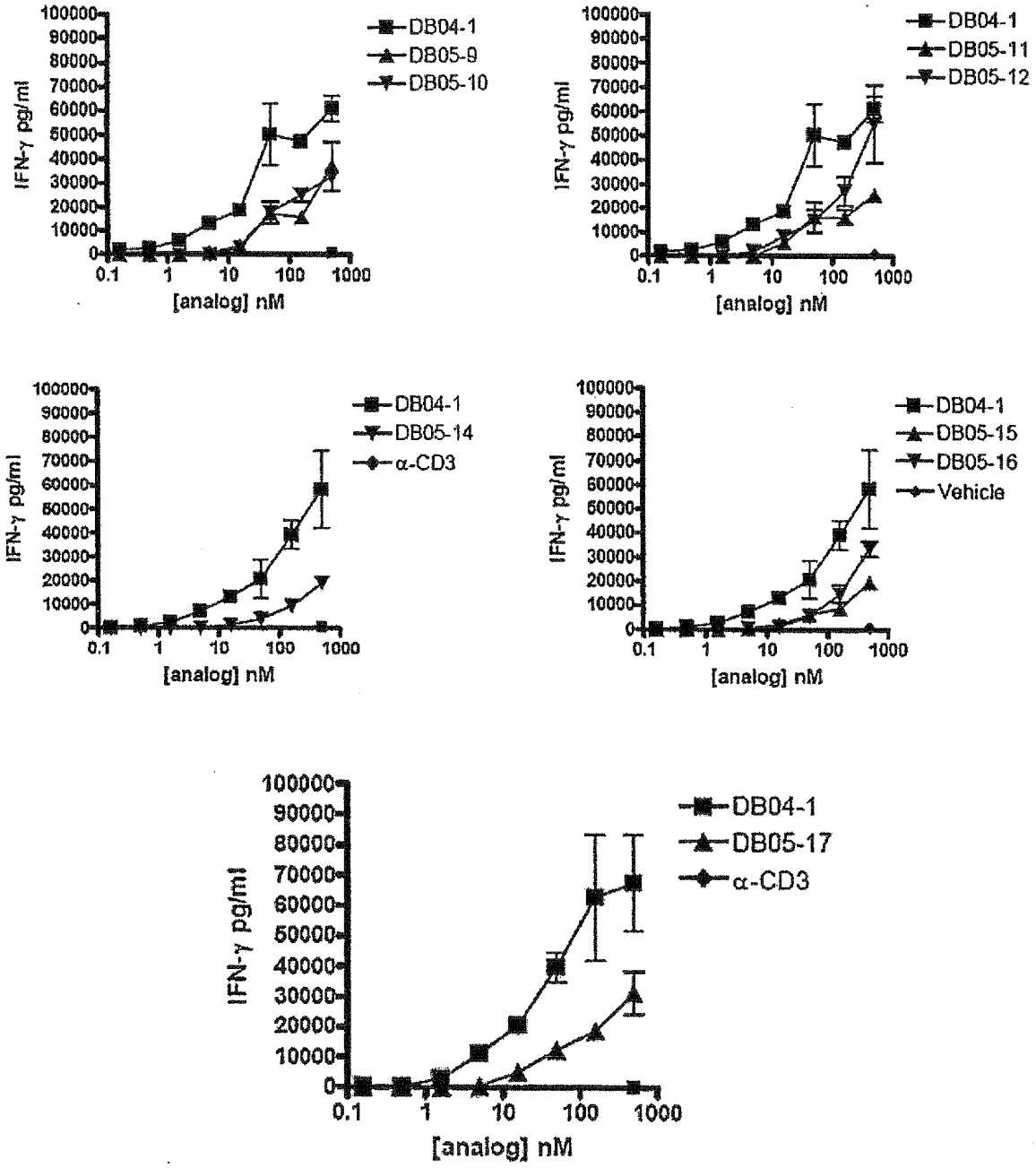

IFN-γ pg/ml
[analog] nM
DB04-1
DB05-9
DB05-10
DB05-11
DB05-12
DB05-14
α-CD3
DB05-15
DB05-16
Vehicle
DB05-17

FIG. 13

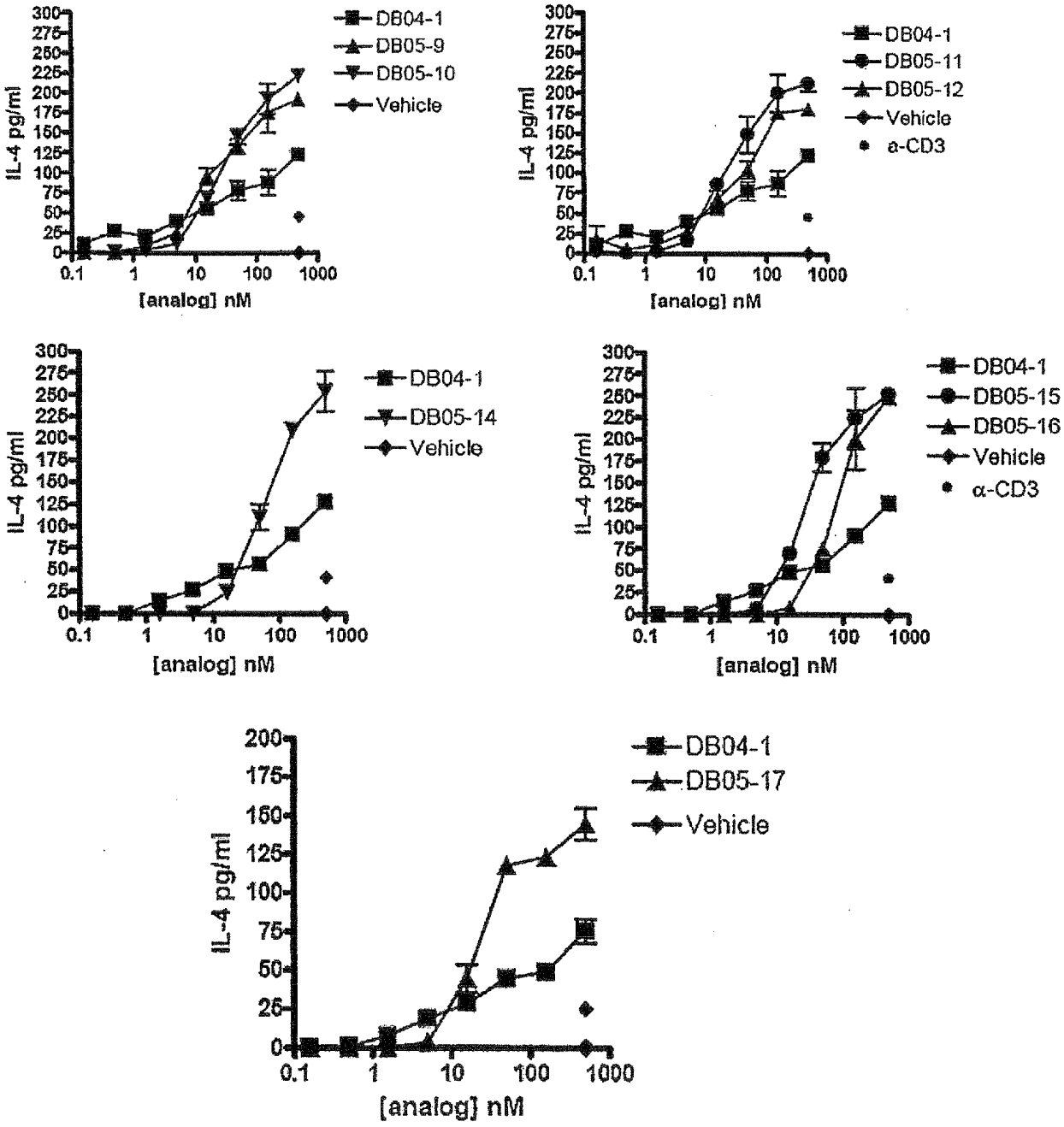
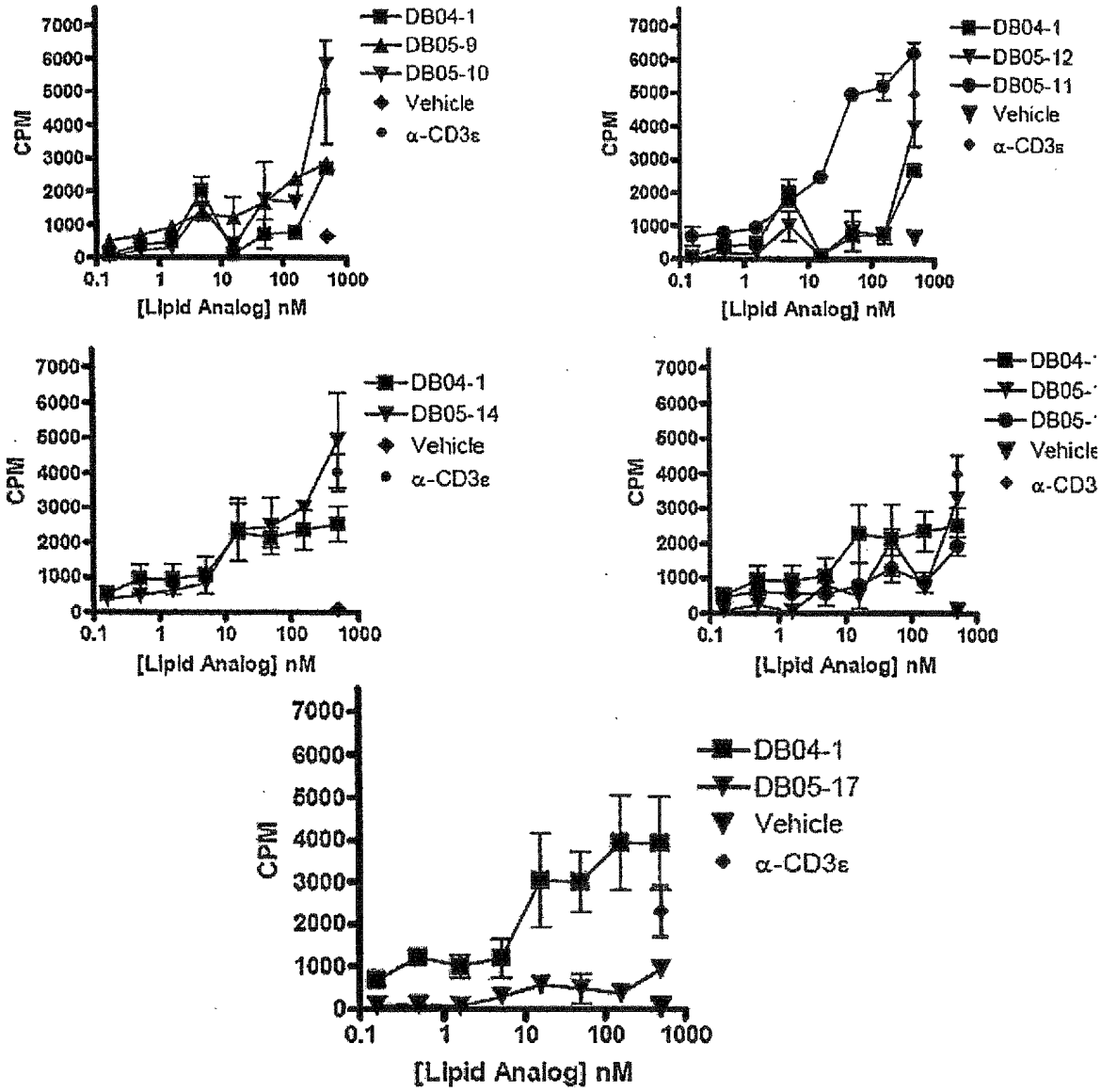

IL-4 pg/ml
[analog] nM
DB04-1
DB05-9
DB05-10
Vehicle
DB04-1
DB05-11
DB05-12
Vehicle
a-CD3
DB04-1
DB05-14
Vehicle
DB04-1
DB05-15
DB05-16
Vehicle
α-CD3
DB04-1
DB05-17
Vehicle

FIG 14


CPM
[Lipid Analog] nM
DB04-1
DB05-9
DB05-10
Vehicle
α-CD3ε
DB04-1
DB05-12
DB05-11
Vehicle
α-CD3ε
DB04-1
DB05-14
Vehicle
α-CD3ε
DB04-
DB05-
DB05-
Vehicle
α-CD3
DB04-1
DB05-17
Vehicle
α-CD3ε

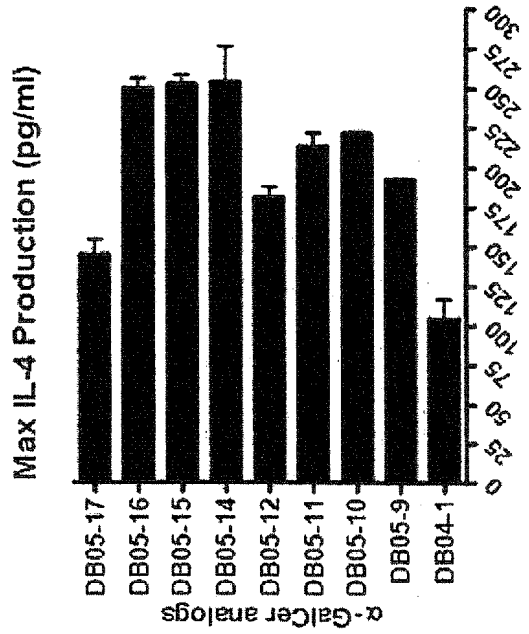
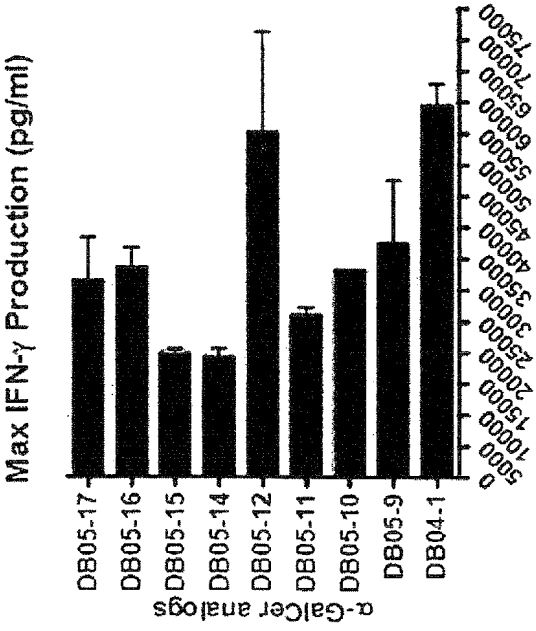

Max IL-4 Production (pg/ml)
0
25
50
75
100
125
150
175
200
225
250
275
300
DB05-17
DB05-16
DB05-15
DB05-14
DB05-12
DB05-11
DB05-10
DB05-9
DB04-1
α-GalCer analogs

FIG. 15


Max IFN-γ Production (pg/ml)
0
5000
10000
15000
20000
25000
30000
35000
40000
45000
50000
55000
60000
65000
70000
75000
DB05-17
DB05-16
DB05-15
DB05-14
DB05-12
DB05-11
DB05-10
DB05-9
DB04-1
α-GalCer analogs

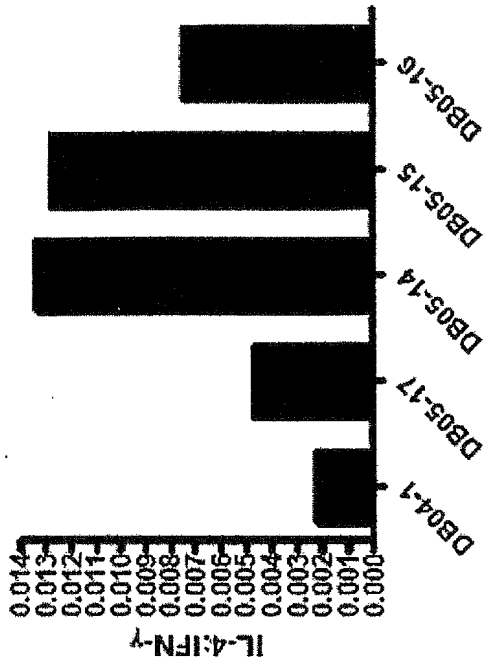
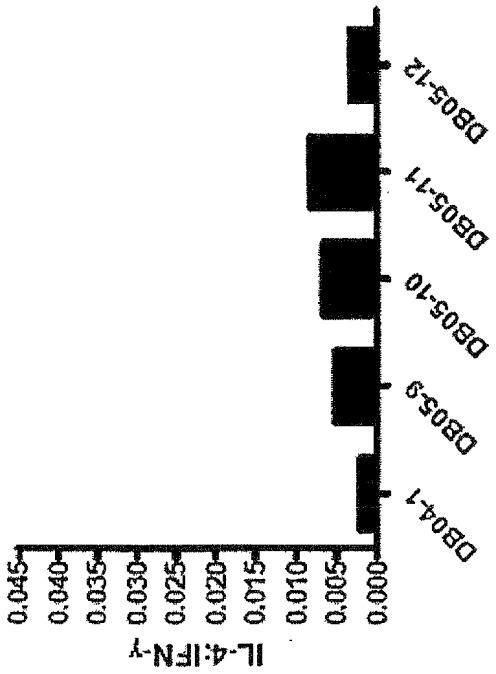

DB05-16
DB05-15
DB05-14
DB05-17
DB04-1
0.014
0.013
0.012
0.011
0.010
0.009
0.008
0.007
0.006
0.005
0.004
0.003
0.002
0.001
0.000
IL-4:IFN-γ

FIG. 16


DB05-12
DB05-11
DB05-10
DB05-9
DB04-1
0.045
0.040
0.035
0.030
0.025
0.020
0.015
0.010
0.005
0.000
IL-4:IFN-γ